SHORT COMMUNICATIONS

DOI: 10.7589/JWD D-25-00216

Journal of Wildlife Diseases, 00(00), 2026, pp. 000–000

Toxoplasma gondii: Challenges and Perspectives in Interpreting Longitudinal Seroprevalence Data for a Chronic Parasitic Infection

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ABSTRACT: *Toxoplasma gondii*—the causative agent of toxoplasmosis—is a zoonotic pathogen of warm-blooded hosts. Infection causes mild-to-severe symptoms, including lethargy, fever, muscle pain, abortion, ocular disease, and encephalitis. *Toxoplasma* affects many vertebrate species, although felids are the only known definitive hosts. Seroprevalence in wildlife is often assessed using cross-sectional data, but few studies have tracked individual-level infections through time. We present a 4-yr dataset from white-tailed deer (*Odocoileus virginianus*) with repeated sampling of individuals that highlights challenges associated with assigning serostatus to individuals. Using a modified agglutination test, we observed seroconversion from seronegative to seropositive within individuals, as expected. Although toxoplasmosis is known to be a chronic disease, we also found reversion from seropositive to seronegative. Accurate assignment of serostatus is necessary for evaluating effects of infection on behavioral and physiologic outcomes. However, longitudinal data from individuals whose titers oscillate around the positive threshold present novel challenges. Therefore, we discuss the implications for assigning serostatus for chronic toxoplasmosis infection for three proposed approaches: 1) ever positive, always positive; 2) negative until positive and then always positive; and 3) status by sampling period. Clarifying which approach is used to assign serostatus when analyzing longitudinal *T. gondii* data may enable more meaningful comparisons across systems and studies.

Key words: Toxoplasmosis, chronic infection, longitudinal data, modified agglutination test.

Toxoplasmosis is caused by *Toxoplasma gondii*, a zoonotic protozoan parasite capable of infecting most warm-blooded species (Dubey 2021a). Infection with *T. gondii* occurs through the ingestion of infectious oocysts in the environment, encysted bradyzoites in infected muscle tissue, or via tachyzoites transmission across the placenta and through the blood (Dubey 2021a). Felids serve as the only definitive hosts and are the sole contributor of infectious oocysts into the environment. After infection, *T. gondii* can reproduce asexually in naïve intermediate hosts during two main phases. During phase 1, an acute infection occurs that is driven by rapidly dividing and widely disseminating tachyzoites. This stage often prompts a host immune response, resulting in symptoms such as fever, lethargy, and muscle aches (Dubey 2021a). After several replication cycles, phase 2 begins: tachyzoites invade tissues, differentiate into bradyzoites, and encyst in the central nervous system and striated muscle tissues (Dubey 2021a). This stage has limited treatment options and little is known about cyst clearance, rendering *T. gondii* infection a chronic condition that can recrudesce in immunocompromised hosts (Dubey and Jones 2008).

The clinical and subclinical outcomes of *T. gondii* infection vary widely depending on host immune function and reproductive status. These outcomes are best documented in humans, with immunocompromised and pregnant individuals at highest risk and asymptomatic infections observed in otherwise healthy individuals (Dubey and Jones 2008). By contrast, morbidity and mortality due to toxoplasmosis in wild animal populations are rarely examined or are difficult to distinguish from other contributing factors. However, clinical outcomes can include loss of pregnancy (Fisk et al. 2023), respiratory disfunction (Casagrande et al. 2015), emaciation (Corpa et al. 2013), neurologic disease (Flegr 2015), and mortality (Dubey et al. 2021; Denk et al. 2022).

Toxoplasma gondii infection has been associated with behavioral changes in wildlife, such as reduced risk aversion and predator avoidance (Johnson and Johnson 2021; Nava et al. 2023). These effects may vary across host taxa due to differences in parasite life stage exposure, immune response, and ecologic processes, although these processes have not been formally explored. One hypothesis is that behavioral changes may reflect evolutionary pressure favoring transmission to felids, the parasite's definitive hosts (Dubey 2021a). Thus, behavioral manipulation may be most pronounced in hosts that facilitate transmission to felids, although hosts that are uncommon prey for felids may still show altered behavior depending on cyst location (central nervous system tissue or other; Johnson and Johnson 2021; Nava et al. 2023).

The location and timing of cyst development—and thus, the potential for physiologic, biologic, and behavioral changes—are poorly understood. Acute immune responses appear conserved across species (Dubey 2021b), but the transition to chronic infection and the persistence or clearance of cysts vary. Evidence for cyst clearance exists in laboratory settings (mice [*Mus musculus*]; McGovern et al. 2020; Suzuki 2021) and livestock (cattle [*Bos taurus*]; Dubey 1986), and some evidence for clearance may exist for wild ungulates (elk [*Cervus canadensis*]; Kolören et al. 2019); however, it remains unclear whether clearance potential and mechanism translate across host species. These uncertainties complicate the interpretation of in situ serologic results from wild populations, where infection stage may influence serostatus. For example, during the chronic phase (i.e., when the parasite is relatively inert in tissue cysts), antibody levels may drop below detection thresholds (Robert-Gangneux and Dardé 2012). Conversely, in the acute phase, tachyzoites may circulate in the absence of detectable antibodies (Contopoulos-Ioannidis and Montoya 2018). A better understanding of the timing of cyst formation and antibody dynamics is critical for accurately determining infection status.

Currently, diagnostic tests for serologic surveillance of *T. gondii* include the modified agglutination test (MAT), direct agglutination tests, indirect fluorescent antibody test, and ELISA. The MAT is widely used and is valued for its cross-species applicability (Shaapan et al. 2008; Fernandes et al. 2019) because it does not require species-specific conjugates. Of note, serologic tests detect antibodies (usually immunoglobulin G) indicating prior exposure to a parasite or pathogen and do not necessarily indicate active infection. However, *T. gondii* infection is assumed to be chronic given the limited evidence for tissue cyst clearance. Thus, seropositivity is often considered synonymous with chronic infection in this system.

Interest in *T. gondii* infection in wildlife stems from both ecologic and human health perspectives (Jenkins et al. 2015), because it is a zoonosis transmitted through consumption of oocysts in contaminated food or water, or tissue cysts in meat (Jenkins et al. 2015). Frequently, wildlife samples tested for *T. gondii* are obtained opportunistically—such as postmortem sampling from hunter harvest. Samples from harvested or road-killed wildlife may overestimate prevalence if altered behaviors increase susceptibility (Nava et al. 2023). Even when antemortem samples are available, these samples often reflect infection prevalence at a single snapshot in time. With new wildlife disease surveillance efforts and large-scale, multiyear operations becoming more common, opportunities for acquisition of longitudinal antemortem data from a single individual have become more feasible.

Our research used long-term capture data from a free-ranging white-tailed deer (*Odocoileus virginianus* [WTD]; hereafter, deer) population in southern Texas, USA. We captured deer annually between October and November 2020–23, with certain individuals with GPS collars recaptured across years. This site had a sympatric population of bobcats (*Lynx rufus*) and was largely free of domestic cats. We tested deer ($n = 59$) captured repeatedly for *T. gondii* infection by using a commercially available MAT (Cornell Animal Health Diagnostic Center, Ithaca, New York, USA). We considered a seropositive status designated by a titer $\geq$1:25 (Dubey 2021a; Meyer et al. 2022) and observed six events of seroconversion from seronegative to seropositive

within individuals, as expected. However, in nine deer, we observed titers shifting from seropositive to seronegative status, and in some cases, back to seropositive (Fig. 1). These transitions present challenges in interpreting results predicted by cross-sectional serostatus. For example, one animal (WTD17) would have been considered seropositive in both 2021 and 2022, but seronegative in 2023. This result raises several questions. Does a seronegative result preceding a seropositive result truly indicate seroconversion (as currently assumed), or is it a sampling artifact that could be overturned with additional sampling? Does a seronegative result following a seropositive result indicate that the first result was a false seropositive, or that the second result was a false negative, or a genuine change in infection stage (i.e., cyst formation during chronic infection)? What are the patterns of antibody production with respect to exposure to different parasite life stages, frequency of exposure, and host physiologic stressors? In captive wildlife, domestic livestock, or human studies, one can resample individuals with conflicting results; however, for in situ wildlife research, resampling is more challenging.

We identified two major challenges in using these data: 1) assigning individual-level serostatus when titers fluctuate through time and 2) estimating reliable population-level prevalence from shifting statuses. To address these challenges, we proposed three approaches for assigning individual infection status for longitudinal *T. gondii* seroprevalence data, acknowledging the limitations of each.

The first method—ever positive, always positive—assigns seropositive status to any individual that has ever tested seropositive, across all sampling periods (Fig., approach 1). This approach reduces the specificity of the disease assignment by allowing for more false positives before infection and thus may dilute the effect of infection on behavioral and physiologic outcomes. It also limits the ability to address within-host infection dynamics over time. Also, in this approach, individuals cannot serve as their own controls, with a pre- and postinfection period. Assuming that false positives are not systematically different from true negatives, this conservative approach would dilute rather than exaggerate observed effects, potentially underestimating the magnitude of infection effects. False positives are inherent to any serologic test, and their influence is amplified under this classification scheme.

The second approach (most broadly used in veterinary and human studies) treats individuals as negative until their first positive test, after which they are considered chronically infected: negative until positive and then always positive (Fig. 2, approach 2). This approach allows for within-individual comparisons—using prepositive samples as controls—but assumes chronic infection after the first positive, regardless of antibody fluctuations or tissue status. This approach may also inflate positives, because if a test is a false positive, all subsequent tests will be considered positive when they may not be. This assumption also has food safety implications if seropositive animals are misclassified, because seroprevalence often exceeds tissue prevalence in wildlife.

The third approach—status by sampling period—assigns serostatus independently at each sampling point, based solely on the test result from that time point (Fig. 2, approach 3). Although this approach allows for dynamic status changes, it may misclassify individuals with fluctuating antibodies and similarly dilute infection effects, and it assumes the possibility for clearance of an infection generally considered to be chronic. Furthermore, assigning a serostatus to events (e.g., an unusual movement or behavior) that occur between two sampling points with conflicting statuses remains a limitation of this approach. This method effectively mirrors the assumptions made in cross-sectional studies and could be used to construct prevalence at different time points that would be comparable with those studies (unlike approaches 1 and 2).

At the population level, shifting individual titer status may influence estimates of prevalence. In our data, when considering the same individuals across years within the same population, we observed two scenarios. In scenario A, titers changed within individuals (including loss of seropositive status), but the overall population prevalence was not impacted (40% in both 2020 and 2021; Fig. 1). In scenario B, seroconversions

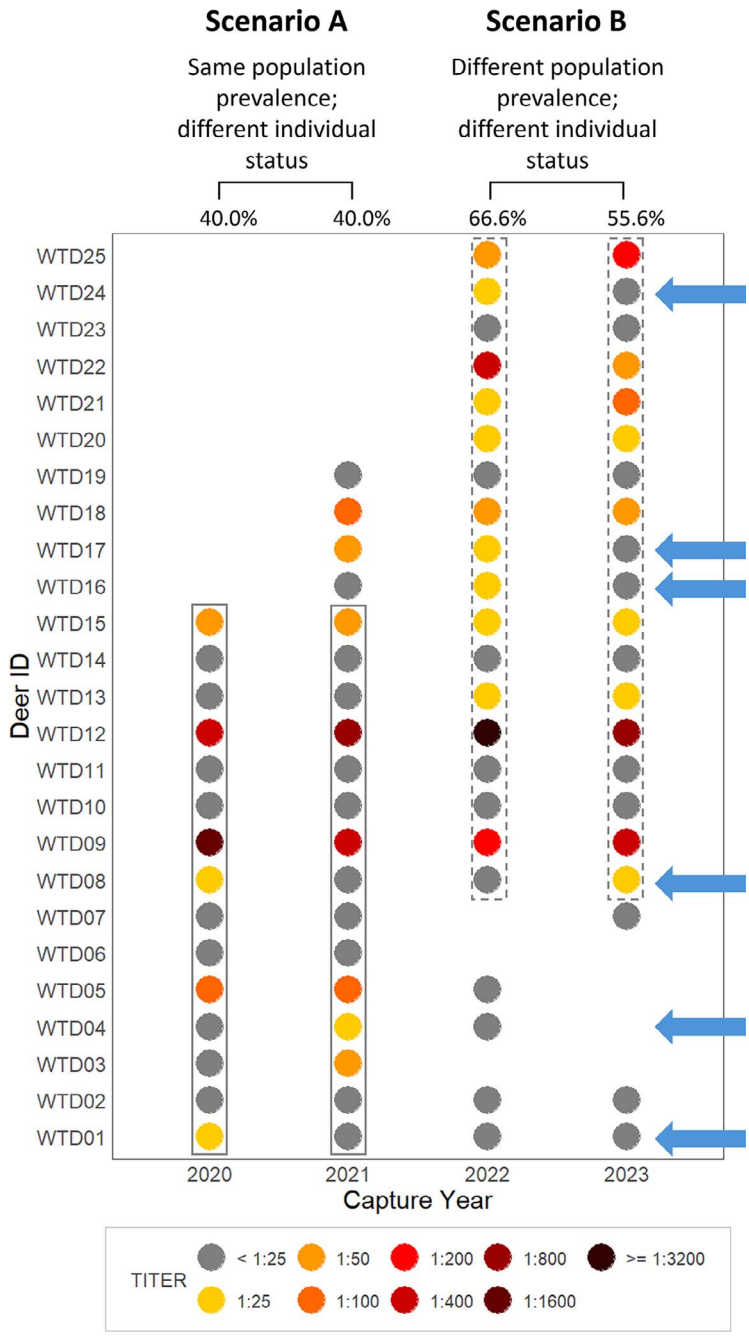

FIGURE 1. Seroprevalence of *Toxoplasma gondii* at the population and individual levels when comparing cross-sectional (i.e., snapshot) data to longitudinal data in white-tailed deer (*Odocoileus virginianus*) on southern Texas, USA, rangelands during 2020–23. In 2020 and 2021 (scenario A), when the same individuals are considered (designated by solid outlined gray boxes), the population prevalence of *T. gondii* remains constant, despite individual statuses changing between years. When comparing a second cohort of individuals between 2022 and 2023 (scenario B; individuals designated by dashed outline gray boxes), both population prevalence and individual disease status change. Some individuals transitioned status, i.e., titers shifted from above the *T. gondii* infection threshold to below, across the 2 yr considered in scenarios A and B (designated by blue arrows).

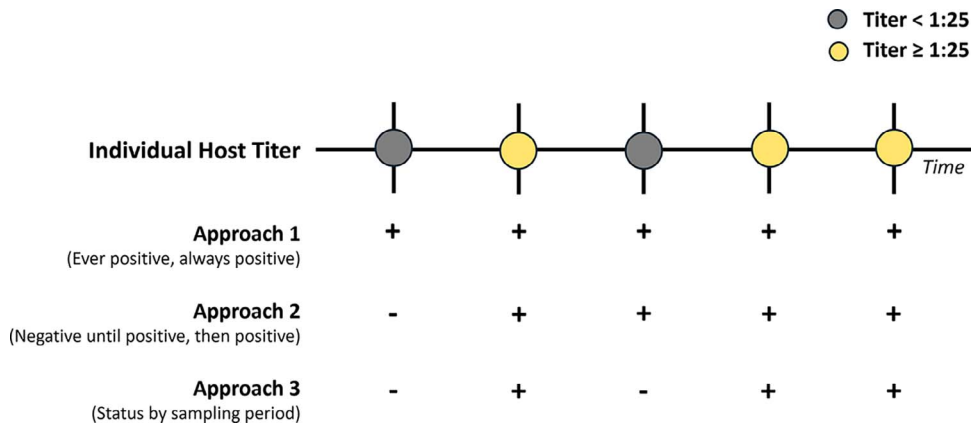

FIGURE 2. Hypothetical assignment of *Toxoplasma gondii* serostatus to an individual with fluctuating serum titer levels across time given three approaches. Approach 1, all sampling points are considered seropositive because there is at least one sampling period over the individual's sampling history that is seropositive. Approach 2, all sampling points after the first detected seropositive are considered seropositive due to assumptions of chronic infection. Approach 3, each sampling point is independent, and status is determined by the titer at that sampling time.

were observed between 2022 and 2023 and resulted in an 11% difference in population prevalence between the years (66 and 55%, respectively; Fig. 1). Population prevalence at a given time point can be estimated using individual serostatus assigned based on the described approaches; however, some (e.g., approach 1) will probably overestimate the population prevalence.

There is a general consensus that *T. gondii* infection is chronic in most species, and that once seropositive, an individual is considered infected for life. Indeed, *T. gondii* antibodies may persist over long durations (mechanisms described in Vargas-Villavicencio et al. [2022]). Nevertheless, we observed seroconversion from seropositive to seronegative, but the underlying drivers and infection dynamics were unclear. Although *T. gondii* antibodies are generally assumed to persist for life, seroconversion to seronegative may be the result of antibody decay (decrease in antibodies through time after infection). This process remains poorly understood and may be host specific, with limited data available from human neonates (Salomè et al. 2022) and from domestic animals monitored ≤1 yr (Olsen et al. 2021; Dini et al. 2024). Conversely, seroconversion may be related to infection stage; during phase 2, when bradyzoites are encysted, the absence of circulating tachyzoites may reduce ongoing antibody stimulation. A similar pattern occurs in Lyme disease (causative agent *Borrelia burgdorferi*), wherein infected individuals may lack detectable antibodies during certain stages of infection despite exhibiting symptoms (Steere et al. 2008). Finally, host taxa and exposure to different parasite life stages (i.e., oocysts versus bradyzoites in tissue cysts) may influence antibody dynamics. For example, longitudinal studies for antibodies to *T. gondii* in wolves (*Canis lupus*) and arctic foxes (*Vulpes lagopus*)—species that probably become infected through consuming cyst-infected tissue—demonstrated titer fluctuations, but none seroconverted from seropositive to seronegative (Bouchard et al. 2018; Meyer et al. 2022). Beyond biologic mechanisms, inherent variability in assay performance—particularly in wildlife species for which validation may be less rigorous than in domestic or laboratory animals—may produce subtle oscillations around the positive-negative cutoff independent of true changes in infection status, a recognized limitation that often motivates running samples in duplicate or triplicate.

A final consideration in assessing *T. gondii* impacts, especially behavioral shifts, is the timing and location of tissue cyst formation, and links to behavioral and physiologic

changes postinfection. Cysts may develop in neural tissue as early as 3 d postinfection (Webster and McConkey 2010), but formation probably varies depending on age, immune status, and species, and may not correlate intuitively with titer levels. The highest titers might occur immediately following initial infection, but this may precede the onset of behavioral shifts, at which time titers may be low. It is also unclear how behavior may change over time with the dormancy or resurgence of bradyzoite activity; titer fluctuations are often not reported in recrudescence.

Our study highlights the complexities of interpreting longitudinal *T. gondii* seroprevalence data in wildlife. Although there is no standard rule for which approach to apply to a given system, researchers may consider more conservative approaches (approach 1, ever positive, always positive) in endangered or vulnerable populations for which the consequences of misclassifying a seropositive individual are greater (high morbidity, mortality, or both) and more liberal methods (approaches 2 and 3) in populations where the disease has minimal clinical significance or the impact may be of little concern. The observed transition between seropositive and seronegative statuses raises important questions about antibody persistence, cyst and infection clearance, and host immune responses. Understanding *T. gondii* infection and titer dynamics may inform disease surveillance strategies, serologic diagnostic criteria, and approaches to investigate ecologic implications. Future research could assess cyst accumulation and clearance through laboratory experiments, validate serologic trends with direct parasite detection methods, evaluate ecologic implications of infection, and apply latent-state models incorporating test error to improve inference about true infection dynamics in free-ranging wildlife.

We thank the many technicians and student volunteers from Southwest Texas Junior College, Stephen F. Austin State University, Sul Ross State University, Tarleton State University, Texas A&M University, Texas A&M University–Kingsville, Texas State University, and Texas Tech University for helping collect data. The East Foundation research team, A. Reeves, T. Campbell, and J. Sawyer, supported capture and sample acquisition. M. Robinson, J. Haynes, S. Vasquez, A. Lopez, and R. Douglas provided logistical and mechanical support during deer captures. Comments provided by T. Campbell, K. W. Hansen, and A. Tanner improved our paper. This is manuscript number 129 of the East Foundation and 25-122 of the Caesar Kleberg Wildlife Research Institute. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

CRediT Author Statement: Kendall Bancroft: Conceptualization; Investigation; Writing - Original Draft; Project administration. **Connor Meyer, Emily Jenkins, Paul C. Cross**: Conceptualization; Writing – Review and Editing. **Randy DeYoung, Aaron Foley**: Funding acquisition; Writing – Review and Editing. **Miranda Hopper, Kevin Lovasik, Landon Schofield, Bryan Spencer**: Investigation; Writing – Review and Editing. **Michael Cherry**: Conceptualization; Investigation; Writing – Review and Editing; Funding acquisition; Project administration. **Alynn M. Martin**: Conceptualization; Investigation; Writing – Review and Editing; Funding acquisition.

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Submitted for publication 23 December 2025.
Accepted 20 March 2026.